JACC Vol. 22, No. 7
December 1993:1897–901

Increased Levels of Serum Neopterin and Decreased Production of Neutrophil Superoxide Anions in Chronic Heart Failure With Elevated Levels of Tumor Necrosis Factor-Alpha

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Objectives. **The purpose of this study was to examine the role of tumor necrosis factor-alpha and tetrahydrobiopterin and superoxide anion release from neutrophils in severe chronic heart failure.**

Background. **Previous studies have demonstrated elevated production of tumor necrosis factor-alpha and free radical-induced endothelial cell damage in severe heart failure.**

Methods. **Plasma and serum levels of immunoreactive interleukin-1, interleukin-6, interferon-gamma, neopterin and tumor necrosis factor-alpha and the release of superoxide anions from circulating neutrophils both at basal conditions and after triggering with f-Met-Leu-Phe or phorbol 12-myristate 13-acetate were measured in 16 patients with severe heart failure and in 11 healthy control subjects.**

Results. **Circulating levels of tumor necrosis factor-alpha and neopterin were elevated in patients with heart failure compared with values in control subjects. A significant correlation between the two was found. Basal and phorbolester-triggered release of oxygen radicals from neutrophils was not affected in patients with heart failure. However, formylpeptide-stimulated release of oxygen radicals by neutrophils was significantly reduced.**

Conclusions. **Suppressed neutrophil function in patients with heart failure exhibiting elevated levels of tumor necrosis-alpha factor may indicate self-protection against the deleterious effects of neutrophil-derived oxygen radicals. Through induction of tetrahydrobiopterin synthesis (as reflected by increased neopterin), tumor necrosis factor-alpha may affect nitric oxide synthesis.**

(J Am Coll Cardiol 1993;22:1897–901)

Recent evidence suggests that the main mechanism of tissue damage in congestive heart failure may involve endothelial dysfunction induced by oxygen free radicals (1). It was found that free radical production is increased in patients with chronic heart failure (2). In the cardiovascular system, sites of oxygen free radical production might include myocardial tissue, especially the mitochondria of ischemically injured myocytes, and the vascular endothelial cells that possess the enzyme xanthine oxidase (1). Neutrophils may play a significant role in this respect because they produce a number of short-lived reactive oxygen species, including hydrogen peroxide, hydroxyl radicals and superoxide anions (3). Such reactive oxygen species derived from neutrophils may contribute to the impairment of endothelium-mediated vasodilation (1).

For the blood-circulating cells to injure endothelial cells, there must be a chemical signal or chemoattractant to activate adherence and respiratory burst or enzyme release. In ischemic reperfusion injury, such chemical signals have been identified (e.g., complement) (3), and their potency may be augmented by priming agents, including proinflammatory cytokines (4) and, as recently suggested for neutrophils, atrial natriuretic peptide (5). Atrial natriuretic peptide and tumor necrosis factor-alpha, which activates the vascular endothelium to release nitric oxide, are increased in heart failure, notably in patients with neurohormonal activation. Their decline after cardiac transplantation, which correlates with normalization of the impaired endothelium (6,7), suggests the hypothesis that atrial natriuretic peptide and tumor necrosis factor-alpha have a pathogenic role in heart failure.

To elucidate the role of tumor necrosis factor-alpha and the involvement of neutrophils in pathophysiologic abnormalities of congestive heart failure, we studied the secretion of superoxide anion by circulating neutrophils and the levels of serum neopterin, which serve as a marker for tetrahydrobiopterin synthesis (8), in relation to plasma levels of atrial natriuretic peptide and proinflammatory cytokines in patients treated for severe heart failure. Tetrahydrobiopterin is a cofactor required for synthesis of nitric oxide (9).

Methods

Study patients. Sixteen patients with severe heart failure were studied (14 men, 2 women, aged 35 to 85 years, mean age 63 ± 4 years [mean ± SEM]). All had dyspnea or fatigue

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Manuscript received February 17, 1993; revised manuscript received July 12, 1993, accepted July 16, 1993.

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0735-1097/93/$6.00

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at rest or on modest exertion (New York Heart Association functional class III or IV) and a left ventricular ejection fraction <35% (as measured by echocardiography, angiography or radionuclide ventriculography; average ejection fraction 28 ± 2%, range 13% to 35%). The causes of heart failure were coronary artery disease in eight patients, end-stage valvular heart disease in one patient and primary dilated cardiomyopathy in seven patients. All patients were clinically stable at the time of evaluation and had no evidence of active infection, inflammatory disease or cancer. The patients were admitted to the hospital with decompensated heart failure and received constant doses of digoxin or digitoxin and vasodilator drugs, including converting-enzyme inhibitors. The doses of diuretic agents had been titrated before blood sampling so that there was no evidence of edema on physical examination.

The control subjects in this study comprised 11 healthy volunteers (8 men, 3 women, aged 24 to 54 years, mean age 38 ± 2 years). Normalcy was determined by a careful review of history and by physical examination and laboratory analysis of diagnostic variables to exclude hematologic, renal or hepatic dysfunction.

From each of the patients as well as control subjects, forearm venous blood was collected for the measurement of atrial natriuretic peptide, tumor necrosis factor-alpha, interleukin-6, neopterin, interleukin-1, interferon-gamma and superoxide anion release from neutrophils.

Hormone and cytokine assays. Plasma and serum samples obtained from the patients and control subjects were stored at −80°C. Storage time was 2 to 6 weeks. Plasma neopterin (Henning), circulating levels of cytokines (tumor necrosis factor-alpha, interleukin-1-beta, interleukin-6, interferon-gamma) (Medgenix) and atrial natriuretic peptide (Eiken) were determined using commercially available test kits, as described elsewhere (10).

Neutrophil preparation and measurement of superoxide anion release. From the peripheral blood (anticoagulated with ethylenediaminetetraacetic acid) neutrophils were obtained after discontinuous density gradient centrifugation of whole blood on Percoll, as previously described (5), followed by hypotonic lysis of contaminating erythrocytes in a sodium chloride solution. The cell preparations (>95% neutrophils by morphology in Giemsa stains, >99% viable by trypan dye exclusion) were resuspended in Hanks' balanced salt solution. Measurement of the production of superoxide anion (O_2^-) was based on the reduction of ferricytochrome *c* by O_2^-, the specificity of reduction being controlled by its inhibition by superoxide dismutase. Immediately after preparation of neutrophils, 100 μl/well of 2×10^6 neutrophils/ml were immersed in a 160-μmol/liter solution of ferricytochrome *c* (Sigma) in phenol red-free Hanks' balanced salt solution containing 1 μmol/liter of f-Met-Leu-Phe or 50 ng/ml of phorbol 12-myristate 13-acetate (Sigma) or vehicle. To one vertical row, ferricytochrome *c* and 300 U/ml of superoxide dismutase (Sigma) were added. This row served as a blank. The plates were covered with lids and placed in a 37°C humidified incubator with a 95% air-5% carbon dioxide atmosphere. After 20 min of incubation, the plates were transferred to the enzyme-linked immunosorbent assay reader, and absorbances were read at the 550-nm wavelength. The absorbance values at 550 nm were converted to nanomoles of O_2^- based on the extinction coefficient of (reduced minus oxidized) ferricytochrome *c*: Optical density at 550 nm = 21×10^3 mol/liter^{-1} cm^{-1}. Because the vertical light path passing through 100-μl ferricytochrome *c* was 3 ml, the concentration of O_2^- was calculated as Nanomoles of O_2^- per well = Absorbance at 550 nm × 15.87 (5).

Data analysis. All group data are expressed as mean value ± SEM or as box plots. The significance of the differences between the two groups was evaluated by the Mann-Whitney *U* test. The probability calculation of significant correlations was performed by Spearman correlation analysis, and $p < 0.05$ was considered statistically significant. Calculations (11) were performed using the StatView II software package (Abacus).

Results

Plasma levels of polypeptide hormone and cytokines. The plasma and serum levels of atrial natriuretic peptide, tumor necrosis factor-alpha, interleukin-6, neopterin, interleukin-1 and interferon-gamma are shown in Figure 1. The 16 patients with heart failure had higher mean serum levels of tumor necrosis factor-alpha (24 ± 4 pg/ml) than the 11 control subjects (11 ± 2 pg/ml, $p = 0.007$). Serum levels of interleukin-6 were also higher in 11 patients with heart failure (36 ± 13 pg/ml) than in 11 control subjects (4 ± 1 pg/ml, $p = 0.011$). However, serum levels of tumor necrosis factor-alpha and interleukin-6 were not elevated in all patients with heart failure. Ten patients had serum levels of tumor necrosis factor-alpha ≥15 pg/ml, whereas 6 patients had serum levels <15 pg/ml. Serum levels of interleukin-6 were >7 pg/ml in 10 of the 11 patients studied. Correlation analysis of tumor necrosis factor-alpha and interleukin-6 in the patients as well as in all control subjects studied did not indicate statistical significance ($n = 11$, $p > 0.05$). Because it is a common finding that one cytokine induces another (12,13), plasma levels of cytokines other than tumor necrosis factor-alpha and interleukin-6 were also measured. Data are available for 11 patients and 11 healthy control subjects. The patients with heart failure did not have elevated serum levels of interleukin-1 (6 ± 1 pg/ml in patients vs. 5 ± 0.1 pg/ml in control subjects, $p > 0.05$) or interferon-gamma (3 ± 0.5 U/ml in patients vs. 2 ± 0.2 U/ml in control subjects, $p > 0.05$).

Plasma levels of neopterin. Plasma levels of neopterin were elevated (≥12 nmol/liter) in 12 of 16 patients, whereas all control subjects had levels <12 nmol/liter (23 ± 4 nmol/liter in 16 patients vs. 9 ± 0.5 nmol/liter in 11 control subjects, $p = 0.0006$). Fourteen of 16 patients had levels of serum neopterin above the upper limit of normal, as previously published (8), for either men or women and adjusted for age. Significant positive correlation was found between

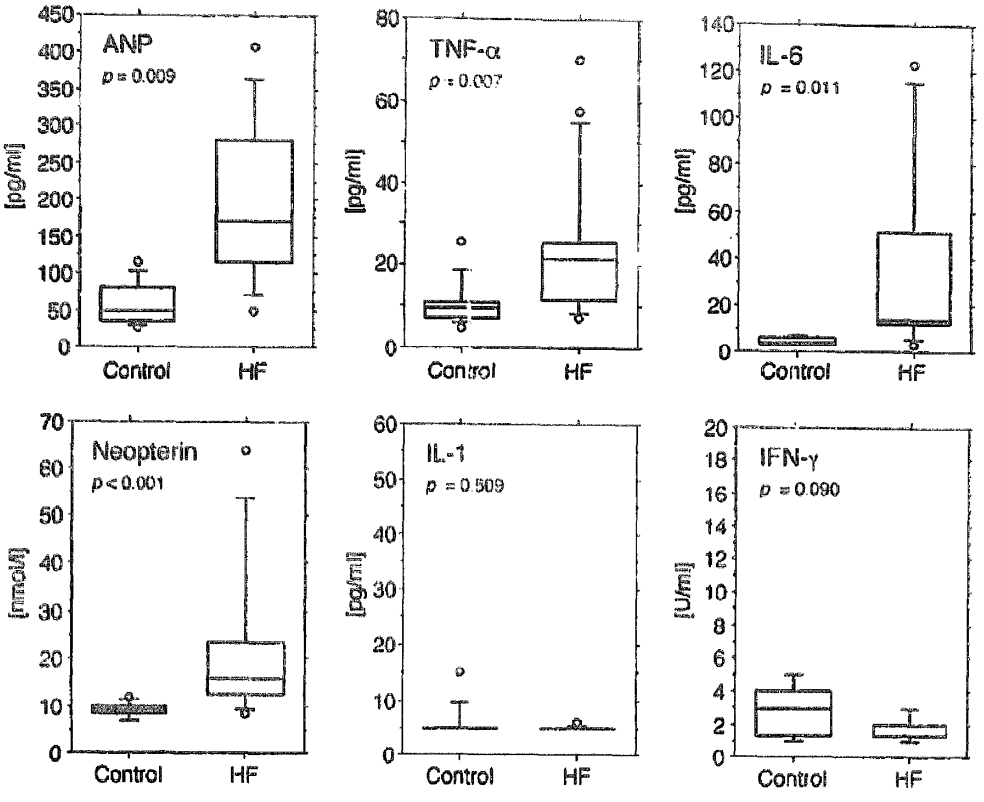


Figure 1. Box plots of plasma and serum levels of atrial natriuretic peptide (ANP), proinflammatory cytokines and neopterin in patients with severe heart failure (HF) (atrial natriuretic peptide, interleukin-6 [IL-6], interleukin-1 [IL-1] and interferon-gamma [IFN-γ], n = 11; tumor necrosis factor-alpha [TNF-α] and neopterin, n = 16) and 11 healthy control subjects (Mann-Whitney *U* test).

levels of neopterin and tumor necrosis factor-alpha (Fig. 2). No correlation was found between neopterin or any of the polypeptide or cytokine levels and hemodynamic variables (not shown).

Neutrophil superoxide anion release. Neutrophils obtained from forearm venous blood were isolated and tested for basal and formylpeptide- and phorbolester-triggered release of superoxide anions in 11 patients and 11 healthy control subjects. Basal reduction of ferricytochrome *c* in the supernatants assayed was low and not significantly different between the patient groups and control groups (optical density at 550 nm was 0.035 ± 0.005 in control subjects vs. 0.024 ± 0.005 in patients, $p > 0.05$). Furthermore, no significant correlation between basal superoxide anion release and plasma levels of any of the cytokines, atrial natriuretic peptide or neopterin was found (data not shown).

The results of formylpeptide- and phorbolester-triggered superoxide anion release are shown in Figure 3. The addition of phorbolester or formylpeptide triggered reduction of ferricytochrome *c* by neutrophils. Comparable amounts of superoxide anion were released from neutrophils in patients and control subjects when phorbolester was used as the triggering agent (3.33 ± 0.22 nmol/well in patients vs. 3.66 ± 0.25 nmol/well in control subjects after 20 min of incubation,

Figure 2. Correlation between serum neopterin levels in patients with severe heart failure and plasma levels of tumor necrosis factor (TNF)-alpha (n = 16), interleukin-6 (IL-6) (n = 11) and atrial natriuretic peptide (ANP) (n = 11) (Spearman rank test).

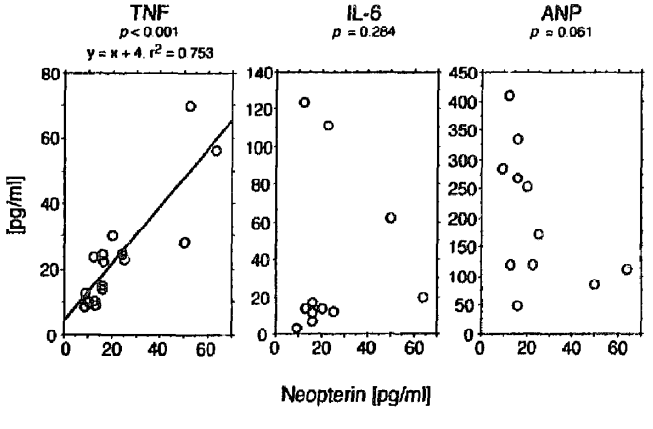
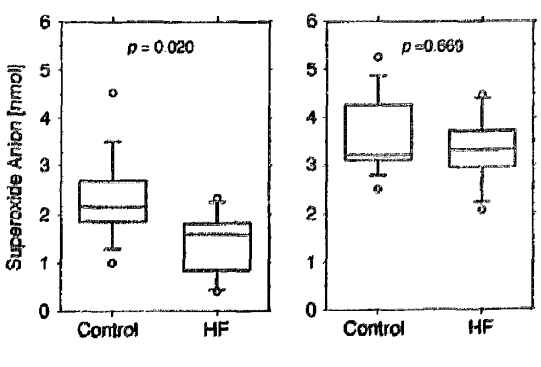


Figure 3. Box plots of f-Met-Leu-Phe–triggered (left) and phorbol 12-myristate 13-acetate–triggered (right) release of superoxide anion from circulating neutrophils in 11 patients with severe heart failure (HF) and 11 healthy control subjects (Mann-Whitney *U* test).



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$p > 0.05$). When formylpeptide was used as the trigger for respiratory burst activation, superoxide anion release from neutrophils was significantly lower in the patient group (1.37 ± 0.20 nmol/well in patients vs. 2.29 ± 0.28 nmol/well in control subjects after 20 min of incubation, $p = 0.020$). Measurement of neutrophil respiratory burst activation in patients and control subjects revealed that there was no correlation with any of the proinflammatory cytokines, atrial natriuretic peptide or neopterin (not shown).

Discussion

Plasma levels of tumor necrosis factor-alpha and neopterin. The findings of this study indicate that plasma levels of neopterin are elevated in patients treated for severe chronic heart failure. Plasma levels of this indicator of cytokine action in vivo (12) have not yet been reported with regard to heart failure. Neopterin levels were elevated primarily in the patients with high levels of circulating tumor necrosis factor-alpha. Tumor necrosis factor-alpha is known to relate to advanced disease in heart failure (6). Levels of tumor necrosis factor-alpha or neopterin were not related to circulating levels of other proinflammatory cytokines, such as interleukin-1, interleukin-6 and interferon-gamma, or atrial natriuretic peptide.

In clinical studies, increased concentrations of neopterin have been reported in various biologic fluids after administration of proinflammatory cytokines, including tumor necrosis factor-alpha. Increased concentrations of neopterin are found also in patients with conditions causing stimulation of cellular immunity with increased activity of mononuclear phagocytes, such as viral or other infections, autoimmune disease and malignancy (13). Neopterin is formed and released by mononuclear phagocytes and other cells during synthesis of tetrahydrobiopterin after activation (e.g., by interferons or by tumor necrosis factor-alpha) (12–14). Because tetrahydrobiopterin is identified as a cofactor for nitric oxide synthase and is utilized by several cell types within the vascular wall (9,15), it is not surprising to find neopterin release in states of cardiovascular alteration in the absence of inflammation, infection or malignancy (16,17).

In cardiovascular physiology, nitric oxide formed from L-arginine, oxygen and reduced nicotinamide adenine dinucleotide phosphate by nitric oxide synthase is a potent endogenous vasodilator and plays a pivotal role in the regulation of vascular tone and blood pressure (1). Correspondingly, elevated levels of serum neopterin have been reported in atherosclerosis (16) and after long distance running (17) but have not been observed after moderate exercise (18). In the case of long distance running, levels of neopterin correlated with elevation of circulating tumor necrosis factor (17). Gross and Levi (9) have reported that tetrahydrobiopterin synthesis is an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. Because in vivo levels of neopterin correspond to the synthesis of tetrahydrobiopterin, our finding in chronic heart failure of elevated levels of neopterin correlating with elevated levels of tumor necrosis factor may reflect increased synthesis of tetrahydrobiopterin induced by tumor necrosis factor-alpha.

All patients had treated, well compensated heart failure at the time of study. Pretreatment cytokine, polypeptide and neopterin levels were not measured. They are likely to differ from levels observed in this investigation because neurohormonal activation in chronic heart failure is affected by previous treatment.

Neutrophil superoxide anion release. Tumor necrosis factor is one of the most potent priming cytokines identified among neutrophil-activating agents in vitro (4). Because release of reactive oxygen radicals from neutrophils is thought to be of importance in cardiovascular pathophysiology, elevation of tumor necrosis factor-alpha levels may thus play an important regulatory role. Therefore, we measured release of superoxide anions from neutrophils in severe heart failure. Our in vivo studies on circulating neutrophils, however, failed to demonstrate an augmented release of superoxide anion from these cells in patients treated for heart failure. Rather, inhibition of f-Met-Leu-Phe–triggered release of superoxide anions from neutrophils was found. Because phorbolester-stimulated release of superoxide anions from neutrophils was unaffected, differences in the age of patients and control subjects probably are not responsible for results observed with formylpeptide as the trigger (19). However, we cannot exclude the possibility that previous pharmacologic treatment as well as the small number and heterogeneity of patients affected the results.

Conclusions. Deactivation of neutrophils in vivo by tumor necrosis factor-alpha has been recognized as a self-protecting mechanism that involves f-Met-Leu-Phe–triggered but not phorbolester-triggered mechanisms (20). Our finding of deactivation of f-Met-Leu-Phe–triggered but not phorbolester-triggered release of superoxide anions from neutrophils in those patients with heart failure who exhibit elevated levels of tumor necrosis factor may be an example of self-protection against the deleterious effects of neutrophils, reflected by an increased release in oxygen radicals. At the same time, via induction of tetrahydrobiopterin, tumor necrosis factor may be involved in the regulation of nitric oxide synthesis, which was found to be impaired in severe heart failure (7,21,22).

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